Coreplication of the major genotype group members of porcine circovirus type 2 as a prerequisite to coevolution may explain the variable disease manifestations

Khaiseb, S ; Sydler, T ; Zimmermann, D R ; Pospischil, A ; Sidler, X ; Brugnera, E

Abstract: A member of the family Circoviridae, porcine circovirus type 2 (PCV2), is associated with postweaning multisystemic wasting syndrome (PMWS), a recent emerging disease worldwide. PCV2 is also found in clinically asymptomatic animals. This paradoxical finding makes the syndrome etiology challenging. We developed new assays to study PCV2 with links to syndrome etiology. For analysis, we used PCV2-infected tissues from subclinically infected and diseased piglets. We compared antigen- and PCV2 DNA-derived signals for tissue localization and intensity. Oligonucleotides were designed to the signature motif of the PCV2 capsid open reading frame to discriminate experimentally between PCV2 genotype groups by PCR, in situ hybridization (ISH), and fluorescence in situ hybridization (FISH). Unexpectedly, all PCV2-infected animals carried both PCV2a and PCV2b genotype group members. Using confocal microscopy, genotype single-cell infections and cell superinfections were visible. Additionally, we discriminated replicative DNA from total PCV2 DNA isoforms with FISH. This aided in our inquiry into cellular genotype-specific replication. Importantly, single-genotype-group replication was not observed. In infected cells with replicating virus, both genotype groups were equally present. These findings suggest PCV2 genotype group members relaxed replication regulation requirements and may even point to PCV2 replication cooperativity in vivo. These observations explain the readily seen PCV2 DNA recombinations and the high overall PCV2 genome plasticity. Hence, we suggest a novel mechanism of syndrome etiology that consists of a continuously changing PCV2 genome pool in hosts and pig herds, posing a constant challenge to the individual maturing immune system.

DOI: https://doi.org/10.1128/JVI.05156-11

JVI Accepts, published online ahead of print on 24 August 2011
J. Virol. doi:10.1128/JVI.05156-11

Porcine circovirus 2 major genotype group members' co-replication as a prerequisite to co-evolution that may explain the variable disease manifestations

Siegfried Khaiseb[1], Titus Sydler[1], Dieter Zimmermann[2], Andreas Pospischil[1], Xaver Sidler[3, 4] and Enrico Brugnera[1, 4, *]

[1]Institute of Veterinary Pathology, University of Zurich, Vetsuisse Faculty, Zurich, Switzerland

[2]Institute of Clinical Pathology, University of Zurich Hospital, Department of Pathology, Zurich, Switzerland

[3]Division of Farm Animals, University of Zurich, Vetsuisse Faculty, Zurich, Switzerland

[4]These authors contributed equally

Running title: PCV2 genotypes superinfections and co-replication

Abstract word count: 247

Text word count: 8201

* Corresponding author. Mailing address: Institute of Veterinary Pathology, Winterthurerstrasse 268, CH-8057 Zurich. Phone: +41 44 635 8576. Fax: +41 44 635 8934. Email: enbrug@vetpath.uzh.ch.

A member of the *Circoviridae* family, porcine circovirus type 2 (PCV2) is associated with postweaning multisystemic wasting syndrome (PMWS), a recent emerging disease worldwide. PCV2 is also found in clinical asymptomatic animals. This paradoxical finding makes the syndrome etiology challenging. We developed new assays to study PCV2 with links to syndrome etiology. For analysis we used PCV2 infected tissue from subclinical infected and diseased piglets. We compared antigen and PCV2 DNA derived signals in tissue localization and intensity. Oligonucleotides were designed to the signature motif of the PCV2 capsid open reading frame to discriminate experimentally between PCV2 genotype groups by PCR, in situ hybridization (ISH) and fluorescence in situ hybridization (FISH). Unexpectedly, all PCV2 infected animals carried both PCV2a and PCV2b genotype group members. Using confocal microscopy, genotype single cell infections and cell superinfections were visible. Additionally, we discriminated replicative DNA from total PCV2 DNA isoforms with FISH. This aided in our inquiry about cellular genotype specific replication. Importantly, single genotype group replication was not observed. In infected cells with replicating virus, both genotype groups were equally present. These findings suggest PCV2 genotype group members relaxed replication regulation requirements and, may even point to PCV2 replication cooperativity *in vivo*. These observations explain the readily seen PCV2 DNA recombinations and the high overall PCV2 genome plasticity. Hence, we suggest a novel mechanism to syndrome etiology that consists of a continuously changing PCV2 genome pool in hosts and pig herds posing a constant challenge to individual maturing immune system.

Members of the *Circoviridae* family and, through interspecies recombination, related viruses cause diseases in vertebrates and plants (14, 20). Viruses' interspecies recombination was

41 linked to disease outbreak (43). *Circoviridae* members PCV2 and its apathogenic PCV1 relative
42 are the smallest autonomous replicating viruses known in eukaryotic cells. PCV2 and PCV1
43 genomic sequences elicit a little less than 80% sequence identity. They are non-enveloped
44 viruses that posses a closed circular, single-stranded DNA (ssDNA) genome (1). Upon infection,
45 the ssDNA genome is converted into a double-stranded (ds) intermediate, the replicative virus
46 DNA isoform (30) or late virus cycle form, which serves as template for rolling-circle synthesis
47 of the viral ssDNA (38). PCV replication occurs by the so-called melting-pot rolling-circle
48 replication mechanism (11). Of note, per one ds PCV2 DNA molecule, one new ss PCV2 DNA
49 is formed (11).

50 The small genome of PCV2 contains at least four open reading frames (ORFs) with
51 known functions: ORF1 codes for two replicase proteins, ORF2 for the structural protein (cap
52 gene) and ORF3 for a protein implicated in cellular apoptosis that overlaps with ORF1 (24, 28).
53 The PCV2 genome is ambisense i.e. the encapsidated viral DNA strand which serves as a
54 template for transcription of the capsid protein gene (ORF2), while the complementary DNA
55 strand of the replicase functions as transcription template for the replicase gene (ORF1) (28).

56 PCV2 emerged in pigs potentially as the result of a cross-species jump from birds into
57 domestic pigs (13), most likely through the wild boar intermediary host in less than a couple of
58 hundred years ago (13). PCV2 adaptation to new hosts is truly remarkable and adaption is not
59 restricted to or ends with pigs as other research groups (19, 39) and we (unpublished data) found
60 PCV2 in bovine. Also, other studies describe PCV2 infections in mouse and human (3, 23, 25).
61 This adaptive virus characteristic is possibly due to the viral genomic plasticity at the mutational
62 rate almost as high as it is described for RNA viruses (13).

Indeed, PCV2 seems the primary etiological agent for PMWS, nevertheless infections are more prevalent than disease and, in animal infection experiments severe clinical signs are rarely manifested. In PCV2 infected animals a transient lymphopenia is often observed and, in neonates, a general depression of the immune system (7, 40). PMWS is an excellent example of disease caused by *Circoviridae*. It is comparably a recent disease as the first cases occurred early 1990s in Western Canada (10). Although PMWS clinical symptoms are well described with wasting and enlarged lymph nodes (35) and microscopic lesions in lymphoid organs by lymphocyte depletion and histocytic infiltrations, the syndrome appears multifactorial triggered with a poorly defined disease etiology (36). Hence, researchers suggested cofactors as essential for PMWS development such as other bacterial or viral infections, over-stimulation or immune-suppression, a general genetic shift in the pig population or pig management changes (27). However, none of these cofactors were evident for the Swiss epizooty (41).

Phylogenetical studies divided PCV2 viruses into two major genotype groups namely PCV2a and PCV2b (37). A simple distinction between PCV2a and PCV2b genotypes was achieved by comparing a distinct stretch of amino acids in the viral capsid protein, the so-called signature motif (6). By this criteria also, PCV2c (9), a third genotype group may be included within PCV2b genotype group. A genetic shift from PCV2a to new emerging PCV2b genotype group was detected in Canadian and USA PMWS outbreaks (4, 6). In analogy, we found a dominance of PCV2b genotype group members during the epizooty although PCV2b genotype group members were also found before the epizooty with dominant PCV2a genotype group members' occurrence (41). Additionally we found, beside the PCV2b genotype group dominance, a subgenotype shift (41) in correlation to the PMWS epizooty. Interestingly, various research groups found further evidence for different virulence of PCV2 variants *in vitro* and in

86 animal infection models (12, 17, 33). Although substantial efforts were undertaken neither
87 genotype group members nor a single member was directly correlated with PMWS disease (2,
88 32).

89 To complicate the mechanism of disease onset, it was seen that PMWS diseased animals
90 might carry multiple PCV2 genotype group members (15, 18). Furthermore, several studies
91 presented sequence evidence for PCV2 genotype group members' recombination (18, 21, 26).
92 Recombination events were observed by amplicate sequencing analysis in both major PCV2
93 ORFs (5, 32). One may surmise the contribution of recombination to PCV2 genomic evolution
94 may be the critical factor to initiate PMWS in an animal's immune system already weakened by
95 a PCV2 caused lymphopenia. As different studies also showed, recombination for other viruses
96 may be linked to zoonoses and severe disease outbreaks (14, 43).

97 In order for virus recombination to take place, at least two different non-competitive
98 viruses' DNA need to intracellularly co-replicate. By PCR, ISH and FISH technologies we
99 studied the presence of major genotype groups in piglets at the organs and cellular levels. In this
100 work, we consistently noticed the presence of both major PCV2 genotype groups in PCV2 pre-
101 epizootic and post-epizootic infected piglets indiscriminately of virus concentration within the
102 animal. We confirmed our previous observations that PCV2a genotype group dominated pre-
103 epizootic and, PCV2b, more specifically PCV2b-CH, during the epizooty. Interestingly, we
104 found superinfections and co-replication of members from both major PCV2 genotype groups.
105 Surprising, however, was the occurrence of PCV2 *in vivo* replication exclusive to cells with both
106 genotype group members although virus genotype group single cell infections were also seen.
107
108

MATERIALS AND METHODS

Ethics Statement. The tissue samples were taken from routine necropsy operations by the Institute of Veterinary Pathology, University of Zurich, and adhered to standards and regulations of ISO / IEC 17025 (Number STS 255). This study was carried out also in strict accordance with the recommendations of the Swiss Federal Veterinary Office (FVO) and according to the Ethical Principles and Guidelines for Experiments on Animals as formulated jointly by the Swiss Academy of Medical Sciences (SAMS) and the Swiss Academy of Sciences (SCNAT).

Samples, Plasmids and recombinant PCV2. Paraffin-embedded archived necropsy tissues from piglets of the years 1981 through 2009 were selected randomly and arranged in different tissue micro array (TMA) blocks. Mostly, secondary lymphoid tissues including spleen, tonsils, lymph nodes and peyer's patches of the ileum were used. The tissues were of piglets from 2 to 4 months of age. We selected and investigated 42 piglet cases and 6 subclinical immuno-histochemically (IHC) negative cases. PMWS cases are defined by IHC (29) as containing medium to high PCV2 antigen and showing histological lesions and clinical disease symptoms. As previously described, the presence of PCV2 antigen was determined by IHC (41). DNA was extracted from the same samples using the Qiagen tissue kit (Hilden, Germany) according to the manufacturer's instructions.

Plasmids for PCV2 and genotype specific PCR controls were constructed by ligation of PCV2a (AF109398 or AY754018) or PCV2b (DQ923523) PCR fragments (137 bp excluding oligonucleotide sequences) (41) encoding the signature motif into pCR®2.1-TOPO® vector

131 (Invitrogen™). Correct inserts were confirmed with *Eco*RI restriction endonuclease (New
132 England Biolabs© Inc.) digestion and sequencing of the constructs.

133 Recombinant PCV2a genotype of AY754016 subgenotype and PCV2b DQ923523
134 subgenotype were constructed by PCR amplification from PCV2 infected fixed and paraffin
135 embedded pig tissue. We used oligonucleotide pairs PCV2forP 5'–GCA AAT GGG CTG CTA
136 ATT TTG CAG-3', PCV2revE 5'- GAA TAA GAA AGG TTA AGG TTG AAT TCT GG-3'
137 and PCV2forE 5'-CCA GAA TTC AAC CTT AAC CTT TCT TAT TC-3', PCV2revP 5'-CAC
138 TTC TTC ACC ATG GTA ACC ATC C-3' for PCR amplification. PCR amplificate were
139 digested with *Eco*RI restriction endonuclease (New England Biolabs© Inc.) and *PflM*I restriction
140 endonuclease (New England Biolabs© Inc.) and ligated into *Eco*RI (New England Biolabs©
141 Inc.) digest calf intestinal alkaline phosphatase (CIP) (New England Biolabs© Inc.) treated
142 pCR®2.1-TOPO® vector (Invitrogen™). Whole PCV2 genotype members were excised by
143 *Eco*RI and re-ligated before introduction into porcine kidney 15 (*PK15*) cell line with
144 lipofectamine 2000 reagent (Invitrogen™). *PK15* cells contained neither PCV1 nor PCV2 before
145 transfection.

146 **DNA preparation and genotype group specific PCRs'.** Total DNA was extracted from
147 6 µm thin slices of fixed and paraffin embedded piglet tissues using the Qiagen tissue kit
148 (Hilden, Germany) according to manufacturer's instructions. PCR amplification of dominant
149 PCV2 sequence from tissue section was previously described (41). PCR amplification of PCV2a
150 genotype group specific sequences were achieved using the forward and reverse primers, PCV2F
151 5'-CGY TGG AGA AGG AAA AAY GGC-3' and PCV2R-A 5'-GTA GTA TTC AAA GGG
152 TAY AGA GAT-3'. For PCR amplification of PCV2b genotype group specific sequences we
153 utilized PCV2F oligonucleotide and a PCV2b-specific primer PCV2R-B 5'-GTA TTC AAA

GGG CAC AGA GMG G-3'. We used PCV2a or PCV2b primer set for genotype group specific sequence amplification, for semi-quantitative analysis and sequencing. PCR reactions were performed in the TGradient (Biometra®) and FastStart Taq polymerase (Roche, Molecular Biochemicals). The PCR products were separated on an agarose gel by electrophoresis and visualised with UV light after staining with ethidium bromide (Fluka, Sigma-Aldrich) or GelRed™ (Chemie Brunschwig AG, Basel).

Oligonucleotides used for In Situ Hybridization (ISH) and Fluorescence In Situ Hybridization (FISH) analysis. Oligonucleotides were on both ends, 5' and 3', either biotinylated or fluorescent labelled with Atto 565 or dyomics 630 (Dy 630) (Microsynth AG, Switzerland) depending on usage. All oligonucleotide probes were designed to recognize sequences of PCV2 ORF2 excluding PCV1 sequence recognition. AB 5'-CCA TCT TGG CCA GAT CCT CCG CCG-3' and ABr oligonucleotide 5'-CGG CGG AGG ATC TGG CCA AGA TGG-3' are both 24mers. The sequence of ABr is exactly reverse, complement to AB oligonucleotide sequence. Both oligonucleotides will recognize all described PCV2a, PCV2b and PCV2c genotype group members. They differ in that AB oligonucleotide (AB sequence identical with sense sequence) -recognizes, in the absence of RNA, single strand (ss) and double strand (ds) PCV2 specific DNA and ABr recognizes exclusively PCV2 dsDNA isoform. And oligonucleotide A 5'-GGG GAC CAA CAA AAT CTC TRT ACC-3' and B 5'-GGC TCA AAC CCC CKC TCT GTG C-3' distinguish between PCV2a and PCV2b genotype groups, respectively. Since B oligonucleotide is more GC rich, A oligonucleotide is 2 bp longer to parallel hybridization conditions. Both genotype specific oligonucleotides were designed over the PCV2 ORF2 signature motif, thus the critical nucleotides are located mostly towards the middle of hybridization sequence (31). Oligonucleotides Ar 5'-GGT AYA GAG ATT TTG TTG

GTC CCC-3' and Br 5'-GCA CAG AGM GGG GGT TTG AGC C-3' were designed to distinguish PCV2a and PCV2b genotype groups in the PCV2 ORF2. Additionally, both are of the complement and reverse sequence of A and B oligonucleotides, hence, in the absence of RNA target they would recognize solely the dsDNA isoform of the virus.

ISH with 5' and 3' biotinylated DNA oligonucleotide probes. Before ISH commencement we manually deparaffinized mounted tissue sections. We used DISCOVERY™ instrument (Ventana Medical Systems) to establish PCV2 specific ISHs'. Experimental protocols were run with help of DISCOVERY™ software. We standardized pre-treatment on formalin-fixed, paraffin-embedded tissue sections by using following RIBOMAP™ reagents: we treated slices 28 min with RIBOPREP™ and 8 min with RIBOCLEAR™ at RT before Protease I (Ventana Medical Systems) incubation for 4 min at 37 °C. Next, the tissue sections were overlaid with corresponding 5' and 3' biotinylated oligonucleotides (Microsynth AG, Switzerland) and hybridization solution consisting of 6x SSC, 5x Denhardt solution, 12% dextran sulphate sodium salt and distilled water. The probes were diluted to a final concentration between 2-10 pmol in hybridization solution dependent on negative controls and signal intensity. Afterward, overlaid slides were subjected to a 4 min denaturing step at 90ºC followed by hybridization for two hours at 37 °C. Slides with mounted tissue were washed in two stringency washes of 2x RIBOWASH™ each at 47 ºC for four minutes. Next, samples were treated with RIBOFIX™ reagent for 24 min to fix stably remaining oligonucleotide probe to target DNA. A washing step automatically followed with 1x reaction buffer (Ventana Medical Systems). For signal detection, tissue sections were incubated for 2 hours with BLUEMAP™ detection kit (Ventana Medical Systems) and Pab-Block (Ventana Medical Systems) that reduced unnecessary background staining. Slides were counterstained using ISH-Red (Ventana Medical Systems) and washed,

dehydrated and mounted.

FISH with 5' and 3' fluorochrome Atto 565 or Dy 630 labelled oligonucleotide probes for laser confocal microscopy. Mycrosynth AG (Switzerland) synthesized 5' and 3' chromophores coupled oligonucleotides with either Atto 565 or Dy 630. ISH protocol on the DISCOVERY™ instrument (Ventana Medical Systems) provided groundwork for FISH experimental steps: both protocol procedures were identical to step with post-fixation with RIBOFIX™ reagent. Separately for FISH, we washed slides twice with distilled water followed by a nuclear staining with 1 µg/ml diamidino-2-phenylindole (DAPI) (AppliChem GmbH, Germany) in methanol for 20 min. After slides were washed again in distilled water and air-dried prior to application of aqueous mounting medium, Immu-Mount (Thermo™, Pitsburgh) and cover slip. We compared oligonucleotide hybridization reactivity to low- or non-PCV2 infected pig tissue and other species, including dog, cat and snake organ sections as controls.

For detection of signals we used a Leica SP5 laser confocal scanning microscope with three lasers and with four confocal fluorescence detectors at the Center for Microscopy and Image Analysis (University of Zurich, Switzerland). We used the diode laser excitation at 405 nm for DAPI signal visualization and the Helium Neon laser excitations at 561 nm or at 633 nm for Atto 565 or Dy 630 detection, respectively.

Confocal pictures were analysed with Imaris 6.3.0 (Bitplane, Scientific Software) a multicolour and 3 D /4 D image processing software.

Mounted tissue sections digested either with DNase or RNase enzyme. ISH protocol on the DISCOVERY™ instrument (Ventana Medical Systems) was adjusted for this procedure and slide mounted tissue sections were subjected to either a desoxyribonuclease I (DNase I) or ribonuclease (RNase) digestion before oligonucleotide hybridization. Consecutive tissue section

slides were preferentially used. In brief, tissue sections were for DNA or RNA digestion overlaid with 300 µl enzyme aqueous buffer solution and incubated at 37 °C in a humidifying chamber for the times indicated. We overlaid concentration of 1-100 U DNase I (Fermentas) per tissue section, which were incubated for 1 hour at 37 °C. Other tissue sections were incubated with RNase cocktail for 1 hour at 37 °C. We used a combination of 83 µg/ml RNase A (Fermentas) and 83 U/ml endoribonuclease RNase H (Biolabs) to digest tissue sections with possible target RNA presence. These enzyme concentrations were chosen at the higher end of concentration recommendations of manufacturer. After tissue slices were washed twice with reaction buffer (Ventana Medical Systems) and introduced into DISCOVERY™ instrument (Ventana Medical Systems) we continued the experiment immediately with the step of oligonucleotide hybridization.

RESULTS

Analysis of PCV2 genotype group distribution. The genetic shift to PCV2b genotype members was obvious, nevertheless no particular PCV2b genotype group member was found to cause disease. Hence, we surmised whether PCV2a genotype group members disappeared epizootic or whether double genotype group infections where common and possibly correlated to disease. Therefore, we first optimized PCV2a and PCV2b template specific PCR methods by construction of respective PCV2 genotype sequence containing vectors. We titrated individual constructs and optimized PCR conditions to detect as low as 10 templates per reaction (Fig. 1). Genotype group specific oligonucleotides were designed within the signature motif to specifically distinguish all known PCV2a from PCV2b subgenotypes. These oligonucleotides are

246 different in 2bp at 3' prime end that prevents cross-reaction amplification products even in
247 template concentrations as high as 10^7- 10^8 templates per reaction (Fig. 1). These template
248 concentrations compare to the highest we found from DNA extractions of PMWS diseased
249 animals.

250 Out of our tissue block collection we selected 48 cases by two criteria (Table): we
251 analyzed cases from the time period before and during the Swiss PMWS epizooty; with no, low
252 or medium to high antigen concentration identified immuno-histochemically (IHC) (Table).
253 These tissue blocks were further re-evaluated by three different PCR methods. A PCV2 specific
254 oligonucleotide set from our recent study allowed us to amplify the dominant PCV2 subgenotype
255 indiscriminately of genotype group affiliation for each case and tissue. PCV1 was not detected
256 by this PCV2 specific PCR amplification. Of note, data indicate that the optimized PCR method
257 is more sensitive than IHC (Table). From real-time PCR data we estimated that a PCR
258 amplification signal was at least two logarithms virus genome more sensitive than PCV2
259 antigen-antibody signals (unpublished data). By amplificate sequencing, we found PCV2b
260 genotype group infections predominately occurring during the epizooty reminiscent to our
261 previous findings: 27% before and 97% during the Swiss epizooty were of the PCV2b genotype
262 group. In fact, all 30 PCV2b group infections from the epizooty harboured PCV2b-CH
263 subgenotype. Separately, for four randomly selected PCV2 infected cases we analysed different
264 organs, including lung, spleen, kidney and lymph node by PCR amplification and sequencing.
265 The same virus variant dominantly infected all PCV2 infected organs of individual animal. By
266 genotype group specific PCR amplification we searched for the presence of PCV2a or PCV2b
267 genotype group member infections. We found that all animals were harbouring both genotype
268 groups including the 6 cases that were IHC negative, indiscriminately from time of case

occurrence. Actually, many PCV2b specific PCR amplificate agarose gel electrophoreses band appeared more intense than the counterpart PCV2a genotype group signals from the same animal during the epizooty and visa versa pre-epizootic. It was puzzling to find both genotype groups in all infected tissue sections especially as no molecular controls indicated any contamination. However, it was yet to be shown whether there was a coincidental contamination.

In situ hybridization (ISH) specificity and sensitivity supports PCR derived data, the presence of both major genotype group members in PCV2 infected animals. We decided to further analyze these 48 cases by ISH (Table). Its advantage over PCR is the signal-defined localization. Moreover, ISH derived signal localization was compared to well-defined antigen staining (Fig. 2). Coincidental contamination from organ environment was readily distinguished by ISH. For optimization of signal intensity we first titrated probes on low to high PCV2 antigen content containing tissue sections. We compared oligonucleotide hybridization reactivity to low- or non-PCV2 infected pig tissue and other species, including dog, cat and snake organ sections (Fig. 3). These negative controls included tissue sections with high concentration of hyaluronic acid, amyloids (Fig. 3g), necrotic (Fig. 3h) and apoptotic tissue (Fig. 3e). Even with 5-fold higher oligonucleotide concentration than what we finally applied in hybridization reaction, we did not find any unspecific reaction signals on tissue sections (Fig. 3a, c, e, g, h). We found reminiscent to antigen tissue localization, PCV2-DNA derived signals centro-follicular and interspersed in surrounding tissue probably caused by infected immigrating macrophages (Fig. 4a). Apart from PCR positive IHC negative tissue sections, signal intensity from low to high PCV2 infected tissues correlated between IHC and ISH (Fig. 2b, c; Table). However, PCV2 DNA dependent signals were not comparable to antigen-derived signals in a few tissues strongly positive for PCV2 antigen in PMWS cases with questionable cellular structural integrity.

292 **Template specificity of signals detected by ISH.** For detailed analysis, we identified the
293 type of nucleic acids recognized by oligonucleotides and whether these ISH signals were the
294 result of the oligonucleotide hybridization to DNA, RNA or both targets. We applied serial
295 dilutions of digestions with corresponding activity to remove DNA or RNA targets. Notably,
296 infection degree varies among a pig's primary or secondary lymph organs. As a consequence, we
297 selected spleen tissue from a PMWS diseased animal with medium antigen concentration. At this
298 PCV2 load, splenic tissue ultrastructure was fully conserved. ISH derived blue staining signals
299 were located mostly in histocytes (Fig. 4).

300 We observed in the Dnase I as well as RNase buffer controls, increased ISH signal
301 intensity throughout the different experiments (Fig. 4b). One hour digestion with 50 U DNase I
302 enzyme obliterated signals completely (Fig. 4c). With less time or lower concentration of DNase
303 I, about 10% blue signals remained (Fig. 4d, e). Tissue sections treated with 1 U DNAse I did not
304 reduce signal intensity (data not shown). Reduction of DNA dependent signals was in contrast to
305 tissue sections treated with a combination of RNase A and RNase H (Fig. 4f). We did not
306 observe any signal intensity reduction in sections digested with the RNase cocktail over buffer
307 control alone. Although this RNase cocktail was sufficient in other experiments to eliminate
308 completely RNA derived signals. Therefore, we concluded that the visualized signals were
309 originating solely from DNA homodimers, namely, oligonucleotide probe and PCV2 DNA.
310 Also, other oligonucleotides specific for PCV2 hybridized DNA template dependent (Fig. 4 and
311 data not shown). Additionally, we verified absence of RNA in tissue sections by two other
312 different methods: by reverse transcriptase PCR from RNA virus infected tissue sections
313 (unpublished data) and, with RNA positive control from Ventana Medical Systems for the
314 detection of mRNA in tissue sections.

315 **Cellular co-localization of PCV2a and PCV2b genotype group members.** Previously
316 we determined PCV2a and PCV2b genotype groups' ratio by PCR amplificate sequencing. Also,
317 ISH hybridization on pre-epizootic pig cases revealed PCV2a genotype group signal dominance
318 over PCV2b genotype group infections and, epizootic PCV2b genotype group infection governed
319 signals. We show such an example of tonsil tissue from prior to (Fig. 5a, b) and during epizooty
320 (Fig. 5c, d).

321 Additionally, we noticed on consecutive tissue sections that often ISH signals from both
322 genotype groups overlapped. We questioned whether comparing consecutive tissue sections'
323 signals might perturb results. Hitherto, we established fluorescence in situ hybridization (FISH)
324 to compare genotype group distribution directly on the same tissue section. For FISH we utilized
325 oligonucleotide sequence and hybridization conditions reminiscent to ISH reactions. Specific
326 oligonucleotides were coupled with either Atto 565 or Dy 630 fluorochrome. The fluorescent
327 colours are heat-stable and clearly distinguishable in laser confocal microscopy. In contrast, the
328 combination Atto 594 and Atto 565 fluorochrom interfered with each other depending on PCV2
329 DNA content in sections. Additionally, we did not use green channels for laser confocal
330 microscopy as we found insurmountable interference with tissue section's auto-fluorescence.
331 Finally, we used a 3 colour staining protocol for confocal microscopy: we utilized DAPI blue
332 fluorescent to visualize nuclei, Atto coupled oligonucleotide B and Dy coupled oligonucleotide
333 A for specific PCV2 genotype group DNA labelling. To expedite analysis we compared sections
334 with the help of tissue micro arrays (TMA). The genotype specific oligonucleotides were
335 designed over the signature motif in the ORF2 to discriminate between all PCV2a and PCV2b
336 genotype group members. The highest sequence variability was chosen around the middle of
337 oligonucleotide hybridization sequence to achieve highest template specificity. Additionally, we

confirmed oligonucleotide specificity on fixed and paraffin-embedded cell pellets transfected either with recombinant PCV2a (Fig. 6a, b) or recombinant PCV2b genotype (Fig. 6c, d). PCV2 infections in general were visualized with oligonucleotide AB in red (Fig. 6). Overlap with genotypic FISH appears yellow (Fig. 6a, d). Fixed cell pellet sections from panels (Fig. 6a, c) were hybridized with genotypic oligonucleotide A and panels (Fig. 6b, d) were hybridized with oligonucleotide B. No cross hybridization was seen with genotypic oligonucleotides (Fig. 6b, c). In the TMA block sections we included PCV1 infected and non-infected cell pellets, which were not recognized by any of the genotypic oligonucleotides.

Also in tissue sections, both genotype groups, PCV2a and PCV2b were found concurrent in the same cell with FISH analysis indicated as yellow signal (Fig. 7c). PCV2 was present mostly in the cell cytoplasm and sometimes intra-nuclear. Nevertheless, genotype group superinfections were found in variable ratios and few cells were single-infected by one genotype group member. Often a slight labelling of PCV2b or PCV2a genotype group was found in the background of the other dominant genotype group. Presence of both genotype groups was particularly interesting in tissue from all six piglets with low PCV2 infections (Table). Cells with double infections always appeared equal or stronger in fluorescence intensity in either channel compared to genotype group single cell infections.

Unexpected intracellular co-replication of the two major PCV2 genotype groups. To identify presence of replicative PCV2 DNA isoform, we designed the complementary reverse oligonucleotide to AB oligonucleotide that we named ABr oligonucleotide. The signals observed were nuclear and cytoplasmic that compared to AB oligonucleotide hybridization signals (Fig. 8; Fig. 2 control sections are 90° clockwise rotated to confocal microscopy pictures of Fig. 8).

We found in all tissue sections more AB oligonucleotide labelled cells compared to ABr based signals and all cells labelled with ABr oligonucleotide were also strongly stained by AB oligonucleotide specific probe. In cells with both fluorescence markers labelling overlapped almost completely (Fig. 8c). Cells with replicative isoform mostly appeared compacted round (Fig. 8b, 9) compared to single AB oligonucleotide stained cells (Fig. 8a, c). We estimated the ratio dependent on tissue from PCV2 general infection to ds PCV2 DNA replicative isoform, from 1 to 1 to about 50 to 1, respectively. In low infected tissues we hardly detected any PCV2 replicative isoform specific signals. The PCV2 replicative isoform was readily detected in sections with moderate to very high viral antigen loads. Additionally, we observed this isoform in lymphoid organs, such as tonsil, peyer's patches of ileum, lymph nodes, and spleen and to a lesser extent in lung and liver (data not shown).

As we found many infected cells carrying both major genotype groups albeit with different ratio, it was of particular interest to understand whether these genotypes might co-replicate intra-cellularly. Hence, we designed a FISH experiment with reverse complement sequence to A and B oligonucleotides, namely with oligonucleotide Ar and Br, which specifically recognizes genotype group specific replicative isoform. Labelling was observed in moderate to high antigen containing tissue sections. Notably, cells with PCV2 replicative DNA isoform were always labelled with both Ar and Br oligonucleotides (Fig. 9). We observed only minor signal intensity differences, if any, between the specific PCV2 genotype groups cell internal replication (Fig. 9c). These co-localized signals rarely varied in contrast to genotype specific A and B oligonucleotide labelling. This pattern was supported in different tissue sections.

DISCUSSION

We demonstrate double genotype group infections in all investigated PCV2 infected pigs and organs. Furthermore, we found *in vivo* cell superinfections and co-replication of the two major PCV2 genotype groups, PCV2a and PCV2b. This explains the recent findings about the PCV2 nature in general and in particular the heightened recombination efficiency of these two major PCV2 genotype groups. To our knowledge this is the first report directly showing superinfections of two so closely related viruses' and suggest genotype groups' cooperation for efficient co-replication *in vivo*. These findings give a new twist to syndrome etiology understanding. A major thinking leap is required to connect PCV2 with PMWS and consequently cofactors are assumed to be essential to trigger disease. In our suggested model no known cofactor is required to initiate PMWS. Instead we suggest presence, co-evolution and autogenic selection of members of both major genotype groups as essential and possibly driven by host's maturing immune system.

We report 100 % of investigated PCV2 infected pigs and their organs carry both genotype groups. These surprising results were confirmed by three unrelated methods: genotype specific PCR, ISH and FISH. Even IHC negative tissue samples with low virus concentration content were found infected with both genotype groups. Although other research had already reported double infections in pigs (6, 8, 15, 18), the prevalence was no higher than 25 % of all infected animals (18). Until our current study, there was no remote suggestion of cell superinfections by both genotype group members. We found retrospectively double infections in tissue samples dating back to 1981. These data indicate a genotype co-localization requirement for PCV2 replication *in vivo* and also support the surprising theoretical notion that PCV2a and PCV2b genotype group members co-evolutionary were transferred from birds to pigs (13).

406 The model needed further evaluation about specificity and sensitivity of applied methods.
407 As research reports about PCV2 recombination heavily relied on sequences derived by PCR
408 method and thus possible artefacts could not be excluded. We checked in depth the genotype
409 group specific PCR amplification sensitivity and specificity. The sensitivity limits were about
410 10-100 templates per reaction and no cross-reactivity of genotype group specific PCRs were
411 found. Also, ISH or FISH signals were dependent on DNA template. Moreover, we found on
412 single genotype group infected porcine kidney cells only oligonucleotide hybridization to
413 corresponding genotype DNA. We surmised whether double genotype infection would enhance
414 PCV2 propagation *in vitro*. Preliminary data indicated that this might not be the case. In this line
415 of thought, we were not able to find any PCV2 double stranded DNA isoform in the single
416 subgenotype infected cell culture. The isoform may be transient or alternatively present in low
417 concentrations in PCV2 infected cell culture. This observation correlates to the generally low
418 propagation efficiency of PCV2 *in vitro*.

419 Previously, we identified 42% of all IHC negative cases by PCR as PCV2 infected (41).
420 We randomly chose six of them for genotypic PCR, ISH and FISH analysis. The signals were
421 confined to histocytes, a cell type generally known to contain virus antigen when infected with
422 PCV2. Also, we observed during epizooty a genetic shift to PCV2b genotype group.
423 Nevertheless**,** data indicate that PCV2b genotype group members were always present and did
424 not newly occur even though its occurrence dominates today's epizooty in Switzerland as well as
425 in other countries including USA and Canada. Even as this genetical shift was observed, every
426 PCV2 infected animal and infected organ was found to carry both PCV2a and PCV2b genotype
427 groups. In fact by PCR amplification and sequencing, others and we identified several members
428 of a particular genotype group in the same animal (15, 41).

We demonstrated the presence of both genotype groups in individual cells. Double genotype group infections per cell were not seen sporadically, on the contrary, they were found even in tissue with very few infected cells. Indeed, superinfections were more prominent in these tissues. These observations were counterintuitive to expectations as the general assumption is that one or the other phylogenetically closely related virus has a slight replication advantage over the other, and consequently one genotype would become diluted and disappears unless there is a special requirement for presence of both genotypes. Alternatively, at least in secondary lymphoid organs these PCV2 carrying cells might be immigrating phagocytes. In our observations, both PCV2 genotype groups were always seen replicating together suggesting that both genotype groups replicate cooperatively *in vivo.* It needs to be seen whether this replication cooperativity can be studied in *in vitro* experiments.

Although we found sporadic single cell infections by PCV2a or PCV2b genotype groups, we observed more cells infected with both genotype group members with varying infection ratios. This pattern was visible not only in the secondary lymph organs, but also in lung and liver tissue. Efficient replication was observed only in cells containing almost equal amounts of the two genotype group members. Affected cells predominantly showed round phenotype, which might be an indication of cellular stress. In neonates, only double genotype infections caused severe disease (16). This supports our *in vivo* findings that effective pathogenicity needs both genotype groups. Some characteristics of PCV2 can also be seen in geminiviruses, where, phylogentically closely related viruses' interspecies recombination correlated with severe disease outbreak (43)**.** However, not all phylogentically closely related viruses allow superinfections (34). The relaxed replication regulation requirements were not previously observed for DNA

451 viruses in general and in particular with closely related viruses. This is truly a new characteristic
452 for PCV2 and needs to also be addressed for other members of the *Circoviridae* family.

453 We identified PCV2a and PCV2b genotype group co-replication. It is easily imagined
454 that within the genotype group co-replication is common and thus facilitates recombination
455 events (22, 32). This also makes sense in the light of virus replication: the virus ssDNA nature
456 and, its propagation by a melting pot rolling cycle mechanism (11). Every new virus appears first
457 as ssDNA template, a replication system known to be exceptionally prone to point mutations.
458 The recent accumulation of reports about PCV2 recombination supports the presence of active
459 replicating genotype and subgenotype members in the cell. This new perspective on the virus
460 enables a rapid changing genetic PCV2 pool in infected cells and pigs, and with it the occurrence
461 of different virulent PCV2 mutants. We suggest the combination of all these factors and
462 particularly the relaxed replication regulation requirement of PCV2 allows the virus to
463 effectively adapt and obviate the host defence system pressure. Additionally, this heightened
464 mutation rate (13) gives the virus a real angle to zoonosis as in less than a couple of hundred
465 years it transferred from birds to the wild boar and into the domestic pig population.

466 Most PCV2 disease research focused on additional cofactors, which would be needed to
467 trigger PMWS disease. However, none of these identified cofactors were of importance at the
468 commencement of the Swiss PMWS epizooty (41). Therefore, we favour a model with a
469 constantly evolving genetic PCV2 pool in cells, in individual animals and pig herds explaining
470 disease etiology. This would further explain the population dynamic of germination centers for
471 PMWS spread and outbreaks in Switzerland and Great Britain as if a new infectious agent with
472 PCV2 co-emerged (42). Under this assumption, the creeping nature of the disease in individual
473 animal during maturation involves harmful virus mutation and recombination that leads to

474 increased lymphopenie that finally triggers disease. Additional cofactors would accelerate
475 disease course in this model. We will, in further animal infection experiments test this
476 hypothesis.
477
478 **ACKNOWLEDGEMENTS**
479
480 We are indebted to Drs. Urs Ziegler and Andreas Kaech and the research team at the
481 Center for Microscopy and Image Analysis (University of Zurich) for all their help. We would
482 like to thank Roseline Weilenmann at the Institute of Veterinary Pathology for her dedicated
483 technical support and Jeanne Peter and Irene Schweizer at the Graphic Department for her
484 professional assistance. We greatly appreciated Dr. Gordon Allen *PK15* cell line and F217
485 monoclonal anti-PCV2 antibody gifts. This work was supported partly by the Julius-Klaus
486 foundation, the Vetsuisse-Faculty and the University of Zurich.
487
488 **REFERENCES**
489
490 1. **Allan, G. M., and J. A. Ellis**. 2000. Porcine circoviruses: a review. J Vet Diagn Invest
491 **12**:3-14.
492 2. **Allan, G. M., F. McNeilly, M. McMenamy, I. McNair, S. G. Krakowka, S. Timmusk,**
493 **D. Walls, M. Donnelly, D. Minahin, J. Ellis, P. Wallgren, and C. Fossum**. 2007.
494 Temporal distribution of porcine circovirus 2 genogroups recovered from postweaning
495 multisystemic wasting syndrome affected and nonaffected farms in Ireland and Northern
496 Ireland. J Vet Diagn Invest **19**:668-673.
497 3. **Bernstein, C. N., G. Nayar, A. Hamel, and J. F. Blanchard**. 2003. Study of animal-
498 borne infections in the mucosas of patients with inflammatory bowel disease and
499 population-based controls. J. Clin. Microbiol. **41**:4986-4990.
500 4. **Carman, S., B. McEwen, J. DeLay, T. van Dreumel, P. Lusis, H. Cai, and J. Fairles**.
501 2006. Porcine circovirus-2 associated disease in swine in Ontario (2004 to 2005). Can Vet J
502 **47**:761-762.
503 5. **Cheung, A. K**. 2007. Homologous recombination plays minor role in excision of unit-
504 length viral genomes from head-to-tail direct tandem repeats of porcine circovirus during
505 DNA replication in Escherichia coli. Arch. Virol. **152**:1531-1539.

6. **Cheung, A. K., K. M. Lager, O. I. Kohutyuk, A. L. Vincent, S. C. Henry, R. B. Baker, R. R. Rowland, and A. G. Dunham**. 2007. Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. Arch. Virol. **152**:1035-1044.
7. **Darwich, L., J. Segales, and E. Mateu**. 2004. Pathogenesis of postweaning multisystemic wasting syndrome caused by Porcine circovirus 2: An immune riddle. Arch. Virol. **149**:857-874.
8. **de Boisseson, C., V. Beven, L. Bigarre, R. Thiery, N. Rose, E. Eveno, F. Madec, and A. Jestin**. 2004. Molecular characterization of Porcine circovirus type 2 isolates from post-weaning multisystemic wasting syndrome-affected and non-affected pigs. J. Gen. Virol. **85**:293-304.
9. **Dupont, K., E. O. Nielsen, P. Baekbo, and L. E. Larsen**. 2008. Genomic analysis of PCV2 isolates from Danish archives and a current PMWS case-control study supports a shift in genotypes with time. Vet Microbiol **128**:56-64.
10. **Ellis, J., L. Hassard, E. Clark, J. Harding, G. Allan, P. Willson, J. Strokappe, K. Martin, F. McNeilly, B. Meehan, D. Todd, and D. Haines**. 1998. Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. Can Vet J **39**:44-51.
11. **Faurez, F., D. Dory, B. Grasland, and A. Jestin**. 2009. Replication of porcine circoviruses. Virol J **6**:60.
12. **Fenaux, M., T. Opriessnig, P. G. Halbur, F. Elvinger, and X. J. Meng**. 2004. Two amino acid mutations in the capsid protein of type 2 porcine circovirus (PCV2) enhanced PCV2 replication in vitro and attenuated the virus in vivo. J. Virol. **78**:13440-13446.
13. **Firth, C., M. A. Charleston, S. Duffy, B. Shapiro, and E. C. Holmes**. 2009. Insights into the evolutionary history of an emerging livestock pathogen: porcine circovirus 2. J. Virol. **83**:12813-12821.
14. **Gibbs, M. J., and G. F. Weiller**. 1999. Evidence that a plant virus switched hosts to infect a vertebrate and then recombined with a vertebrate-infecting virus. Proc. Natl. Acad. Sci. U. S. A. **96**:8022-8027.
15. **Grau-Roma, L., E. Crisci, M. Sibila, S. Lopez-Soria, M. Nofrarias, M. Cortey, L. Fraile, A. Olvera, and J. Segales**. 2008. A proposal on porcine circovirus type 2 (PCV2) genotype definition and their relation with postweaning multisystemic wasting syndrome (PMWS) occurrence. Vet Microbiol **128**:23-35.
16. **Harding, J. C., J. A. Ellis, K. A. McIntosh, and S. Krakowka**. 2010. Dual heterologous porcine circovirus genogroup 2a/2b infection induces severe disease in germ-free pigs. Vet Microbiol
17. **Hasslung, F., P. Wallgren, A. S. Ladekjaer-Hansen, A. Botner, J. Nielsen, E. Wattrang, G. M. Allan, F. McNeilly, J. Ellis, S. Timmusk, K. Belak, T. Segall, L. Melin, M. Berg, and C. Fossum**. 2005. Experimental reproduction of postweaning multisystemic wasting syndrome (PMWS) in pigs in Sweden and Denmark with a Swedish isolate of porcine circovirus type 2. Vet Microbiol **106**:49-60.
18. **Hesse, R., M. Kerrigan, and R. R. Rowland**. 2008. Evidence for recombination between PCV2a and PCV2b in the field. Virus Res **132**:201-207.
19. **Kappe, E. C., M. Y. Halami, B. Schade, M. Alex, D. Hoffmann, A. Gangl, K. Meyer, W. Dekant, B. A. Schwarz, R. Johne, J. Buitkamp, J. Bottcher, and H. Muller**. 2010. Bone marrow depletion with haemorrhagic diathesis in calves in Germany: characterization

of the disease and preliminary investigations on its aetiology. Berl Munch Tierarztl Wochenschr **123**:31-41.

20. **Katoh, H., H. Ogawa, K. Ohya, and H. Fukushi**. 2010. A Review of DNA Viral Infections in Psittacine Birds. J Vet Med Sci
21. **Kim, H. K., Y. Luo, H. J. Moon, S. J. Park, H. O. Keum, S. Rho, and B. K. Park**. 2009. Phylogenetic and recombination analysis of genomic sequences of PCV2 isolated in Korea. Virus Genes **39**:352-358.
22. **Lefebvre, D. J., J. Van Doorsselaere, P. L. Delputte, and H. J. Nauwynck**. 2009. Recombination of two porcine circovirus type 2 strains. Arch. Virol. **154**:875-879.
23. **Li, L., A. Kapoor, B. Slikas, O. S. Bamidele, C. Wang, S. Shaukat, M. A. Masroor, M. L. Wilson, J. B. Ndjango, M. Peeters, N. D. Gross-Camp, M. N. Muller, B. H. Hahn, N. D. Wolfe, H. Triki, J. Bartkus, S. Z. Zaidi, and E. Delwart**. 2010. Multiple diverse circoviruses infect farm animals and are commonly found in human and chimpanzee feces. J. Virol. **84**:1674-1682.
24. **Liu, J., I. Chen, and J. Kwang**. 2005. Characterization of a previously unidentified viral protein in porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. J. Virol. **79**:8262-8274.
25. **Lorincz, M., A. Csagola, I. Biksi, L. Szeredi, A. Dan, and T. Tuboly**. 2010. Detection of porcine circovirus in rodents - Short communication. Acta Vet Hung **58**:265-268.
26. **Ma, C. M., C. C. Hon, T. Y. Lam, V. Y. Li, C. K. Wong, T. de Oliveira, and F. C. Leung**. 2007. Evidence for recombination in natural populations of porcine circovirus type 2 in Hong Kong and mainland China. J. Gen. Virol. **88**:1733-1737.
27. **Madec, F., N. Rose, B. Grasland, R. Cariolet, and A. Jestin**. 2008. Post-weaning multisystemic wasting syndrome and other PCV2-related problems in pigs: a 12-year experience. Transbound Emerg Dis **55**:273-283.
28. **Mankertz, A., R. Caliskan, K. Hattermann, B. Hillenbrand, P. Kurzendoerfer, B. Mueller, C. Schmitt, T. Steinfeldt, and T. Finsterbusch**. 2004. Molecular biology of Porcine circovirus: analyses of gene expression and viral replication. Vet Microbiol **98**:81-88.
29. **McNeilly, F., I. McNair, D. P. Mackie, B. M. Meehan, S. Kennedy, D. Moffett, J. Ellis, S. Krakowka, and G. M. Allan**. 2001. Production, characterisation and applications of monoclonal antibodies to porcine circovirus 2. Arch. Virol. **146**:909-922.
30. **Meehan, B. M., J. L. Creelan, M. S. McNulty, and D. Todd**. 1997. Sequence of porcine circovirus DNA: affinities with plant circoviruses. J. Gen. Virol. **78**:221-227.
31. **Naiser, T., O. Ehler, J. Kayser, T. Mai, W. Michel, and A. Ott**. 2008. Impact of point-mutations on the hybridization affinity of surface-bound DNA/DNA and RNA/DNA oligonucleotide-duplexes: comparison of single base mismatches and base bulges. BMC Biotechnol **8**:48.
32. **Olvera, A., M. Cortey, and J. Segales**. 2007. Molecular evolution of porcine circovirus type 2 genomes: phylogeny and clonality. Virology **357**:175-185.
33. **Opriessnig, T., N. E. McKeown, E. M. Zhou, X. J. Meng, and P. G. Halbur**. 2006. Genetic and experimental comparison of porcine circovirus type 2 (PCV2) isolates from cases with and without PCV2-associated lesions provides evidence for differences in virulence. J. Gen. Virol. **87**:2923-2932.
34. **Ramirez, S., S. Perez-del-Pulgar, J. A. Carrion, M. Coto-Llerena, L. Mensa, J. Dragun, J. C. Garcia-Valdecasas, M. Navasa, and X. Forns**. 2010. Hepatitis C virus

superinfection of liver grafts: a detailed analysis of early exclusion of non-dominant virus strains. J. Gen. Virol. **91**:1183-1188.

35. **Segales, J., G. M. Allan, and M. Domingo**. 2005. Porcine circovirus diseases. Anim Health Res Rev **6**:119-142.
36. **Segales, J., and M. Domingo**. 2002. Postweaning multisystemic wasting syndrome (PMWS) in pigs. A review. Vet Q **24**:109-124.
37. **Segales, J., A. Olvera, L. Grau-Roma, C. Charreyre, H. Nauwynck, L. Larsen, K. Dupont, K. McCullough, J. Ellis, S. Krakowka, A. Mankertz, M. Fredholm, C. Fossum, S. Timmusk, N. Stockhofe-Zurwieden, V. Beattie, D. Armstrong, B. Grassland, P. Baekbo, and G. Allan**. 2008. PCV-2 genotype definition and nomenclature. Vet Rec **162(26)**:867-868.
38. **Steinfeldt, T., T. Finsterbusch, and A. Mankertz**. 2006. Demonstration of nicking/joining activity at the origin of DNA replication associated with the rep and rep' proteins of porcine circovirus type 1. J. Virol. **80**:6225-6234.
39. **Tischer, I., L. Bode, J. Apodaca, H. Timm, D. Peters, R. Rasch, S. Pociuli, and E. Gerike**. 1995. Presence of antibodies reacting with porcine circovirus in sera of humans, mice, and cattle. Arch. Virol. **140**:1427-1439.
40. **Vincent, I. E., C. Balmelli, B. Meehan, G. Allan, A. Summerfield, and K. C. McCullough**. 2007. Silencing of natural interferon producing cell activation by porcine circovirus type 2 DNA. Immunology **120**:47-56.
41. **Wiederkehr, D. D., T. Sydler, E. Buergi, M. Haessig, D. Zimmermann, A. Pospischil, E. Brugnera, and X. Sidler**. 2009. A new emerging genotype subgroup within PCV-2b dominates the PMWS epizooty in Switzerland. Vet Microbiol **136**:27-35.
42. **Woodbine, K. A., G. F. Medley, J. Slevin, A. L. Kilbride, E. J. Novell, M. J. Turner, M. J. Keeling, and L. E. Green**. 2007. Spatiotemporal patterns and risks of herd breakdowns in pigs with postweaning multisystemic wasting syndrome. Vet Rec **160**:751-762.
43. **Zhou, X., Y. Liu, L. Calvert, C. Munoz, G. W. Otim-Nape, D. J. Robinson, and B. D. Harrison**. 1997. Evidence that DNA-A of a geminivirus associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination. J. Gen. Virol. **78**:2101-2111.

630 Table. Comparison of fixed and paraffin embedded piglet tissue sections before and
631 during epizooty by PCV2 specific IHC, ISH and PCR.

	Clinical diagnosis	IHC signal intensity	ISH signal intensity	Dominant PCV2 genotype
Before PMWS epizooty	Subcl. infec.	5 neg.	5 +	a/b (n=2)/(n=3)
		2 +	2 +	a (n=2)
	PMWS	2 ++	2 ++	a (n=2)
		6 +++	6 +++	a/b (n=5)/(n=1)
Piglet cases		15	15	15
During PMWS epizooty	Subcl. infec.	1 neg.	1 +	a (n=1)
		4 +	4 +	b (n=4)
	PMWS	6 ++	2 ++ / 4 +	b (n=6)
		22 +++	5 +++/ 9 ++/ 8 +	b (n=22)
Piglet cases		33	33	33

632

633 PMWS clinical diagnosis, IHC and Swiss epizooty were previously described (41). ISH
634 signal intensity comparisons were achieved with aid of oligonucleotide AB tissue section
635 labelling. The dominant genotype group (a or b) was determined with PCV2 specific PCR
636 amplification and product sequencing. The + indicates staining strength with +++ indicating the
637 strongest label; the staining intensity begins with negative (neg.; no cell was visibly labelled).
638 Subclinically infected (Subcl. infec.) piglets with PCV2 infections were clinically inconspicuous.
639 Numerals or (n=x) indicates number of piglets investigated.

640

641

642 **Figure Legends:**

643

644 Fig. 1. Genotypic PCR specificity and sensitivity. Two panels show genotypic PCR
645 amplificate signals separated by agarose gel electrophoreses. From left to right panels are further
646 divided by DNA ladder into PCR reactions against plasmids containing PCV2a or PCV2b
647 genotype sequence. At the lower bottom, numbers indicate serial template dilutions (10^0-10^7)
648 from corresponding plasmids. On the right side of the panels, the molecular weight of DNA
649 ladder single bands in base pairs (bp) is indicated.

650

651 Fig. 2. HE, IHC and ISH comparison of a moderately PCV2 infected spleen section.
652 From left to right: (a) Heamatoxylin stained section (HE), (b) anti-PCV2 IHC (F217 monoclonal
653 anti-PCV2 antibody clone (29)) and (c) ISH with AB oligonucleotide hybridization against
654 PCV2-DNA. At the right lower bottom of pictures, bar represents 20 µm.

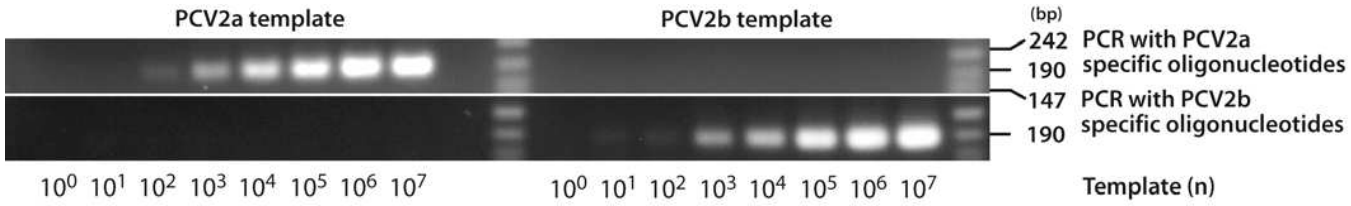
655

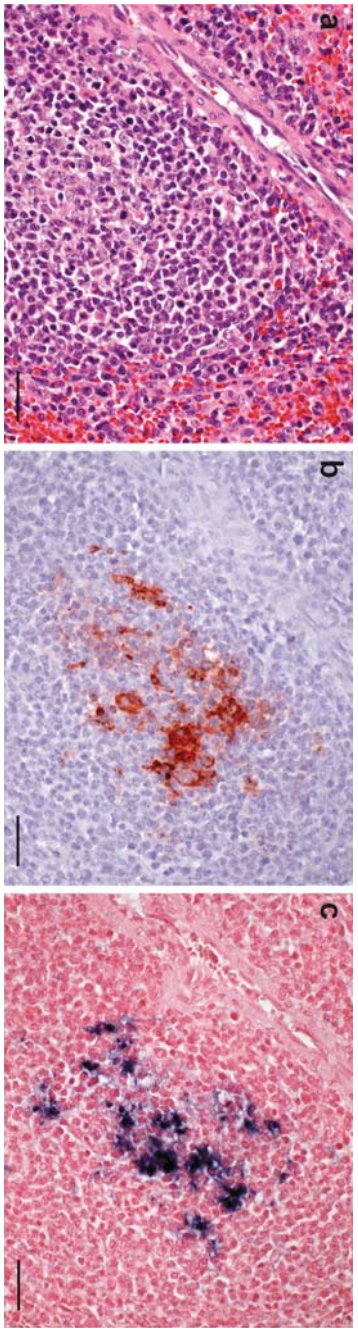
656 Fig. 3. Highly PCV2 infected and non-infected tissue comparison by PCV2 specific ISH
657 oligonucleotide hybridization. We used AB oligonucleotide probe (blue coloration) on
658 previously PCV2 antigen grade (IHC) defined tissue sections from pig liver (a, b), pig lymph
659 node (c, d), dog kidney leptospirosis diagnosis (e), pig tonsil (d), cat liver with amyloidosis
660 diagnosis (g) and snake liver with hemosiderosis diagnosis (h, reptile pigmentation is dark brown
661 and iron deposits are colored light brown). The white letters (b, d, f) correspond to the highly
662 PCV2 infected tissue panels and, black letters (a, c, e, g, h) to ISH negative tissue samples. All
663 tissues were compiled to one TMA block and exposed together to AB oligonucleotide probe

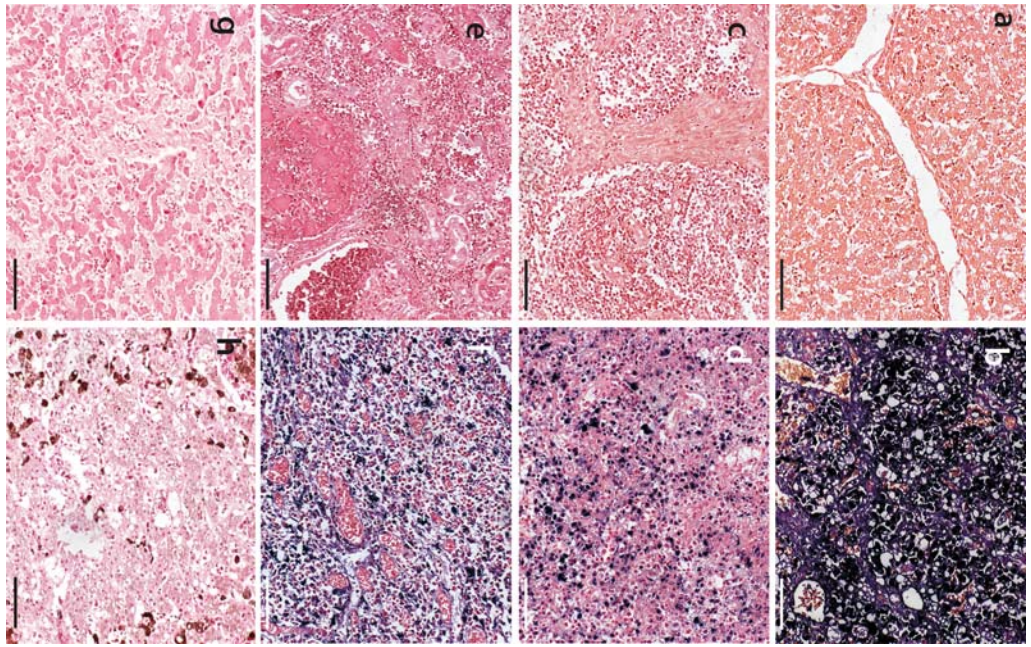
664 hybridization and signal (blue) development. At the right lower bottom of pictures, bar
665 represents 50 µm.
666
667 Fig. 4. Oligonucleotide hybridization target identification on spleen sections from PMWS
668 diseased piglet tissue. Oligonucleotide ISH on consecutive formalin fixed paraffin embedded
669 spleen sections. These tissue sections were either untreated (a) or, for 1 hour incubated with
670 digestion buffer only (b), for one hour digested with 50 U DNase I (c), for 5 min treated with 50
671 U DNase I (d), for one hour digested with 20 U DNase I (e), for one hour incubated with an
672 effective Rnase A/Rnase H cocktail (f) followed by ISH with oligonucleotide AB. Arrows
673 indicate PCV2 target presence (blue signals) in periarteriolar lymphatic sheath and probably in
674 histocytes/macrophages distributed throughout the tissue sections. The tissue sections were
675 counterstained with ISH-Red. At the right lower bottom of pictures, bar represents 50 µm.
676
677 Fig. 5. PCV2 genotype group distribution before and during the epizooty. PCV2a/PCV2b
678 genotype groups differential signals of fixed and paraffin embedded tonsil tissue sections prior to
679 (a, b) and during the Swiss epizooty (c, d). ISH with oligonucleotide A (a, c) and oligonucleotide
680 B probe (b, d) that recognize PCV2a and PCV2b viruses, respectively. Note presence of PCV2
681 genotype group signals by a blue stain also indicated by arrows. Tissue sections were
682 counterstained with ISH-Red. At the right lower bottom of pictures, bar represents 50 µm.
683
684 Fig. 6. Specificity of genotypic oligonucletide scrutinized on recombinant PCV2a or
685 recombinant PCV2b infected cell layers in laser confocal microscopy. Selected panels from a
686 TMA block sections of formalin fixed and paraffin embedded genotype specific transfected

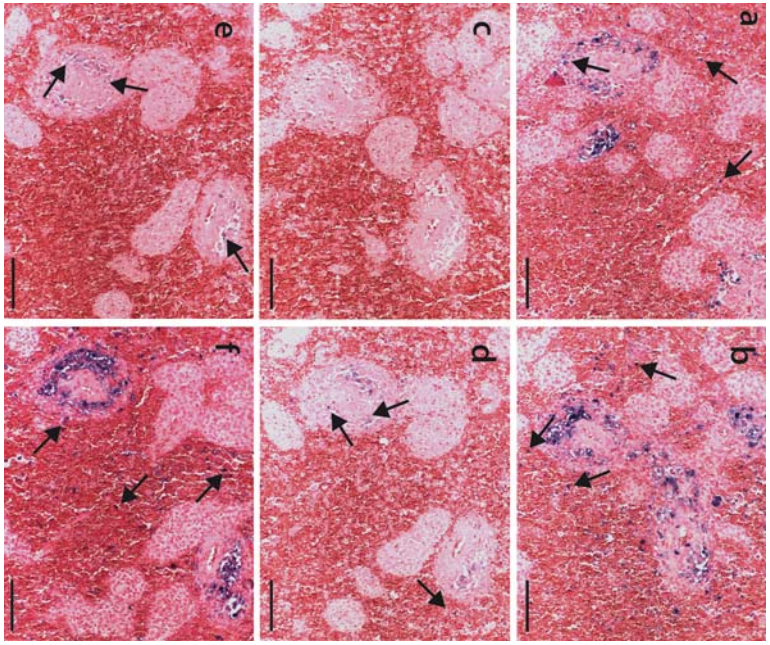
687 *PK15* cell layers. PCV2a or PCV2b genotype infected tissue were either stained with a
688 combination of oligonucleotides AB appearing in red and A that would appear green as single
689 color (a, b) or separately with oligonucleotides AB appearing in red and B that would appear
690 green as single color (c, d). The overlap of both colors, oligonucleotides AB and the genotypic
691 oligonucleotides, appear yellow. Nuclei are counterstained with DAPI and thus appear blue. The
692 bar indicates 80 µm.
693
694 Fig. 7. Laser confocal microscopy analysis revealed PCV2a and PCV2b genotype group
695 infections in the same cell. FISH from fixed and paraffin embedded lymph node tissue section of
696 an epizootic PMWS diseased animal. Red signals derived from the PCV2b specific binding
697 oligonucleotide B (a). Green represents signals achieved from oligonucleotide A target
698 hybridization (b). Panel c results from overlay of (a) and (b) pictures. In all panels nuclei are
699 counterstained with DAPI and thus appear blue. The bar indicates 10 µm.
700
701 Fig. 8. Detection of PCV2 replication by laser confocal microscopy. FISH used
702 specifically for identification of PCV2 dsDNA in PCV2 infected formalin fixed and paraffin
703 embedded spleen tissue. Red signals derived from oligonucleotide AB hybridization identify all
704 PCV2 infection (a). Green labelling was derived from oligonucleotide ABr hybridization (b).
705 Panel (c) is an overlay of pictures (a) and (b). Nuclei are counterstained with DAPI and thus
706 appear blue. The bar indicates 20 µm.
707
708 Fig. 9. PCV2 genotype groups co-localization and co-replication visualized by a confocal
709 micrograph. Genotype groups superfection and co-replication visualized by FISH of formalin

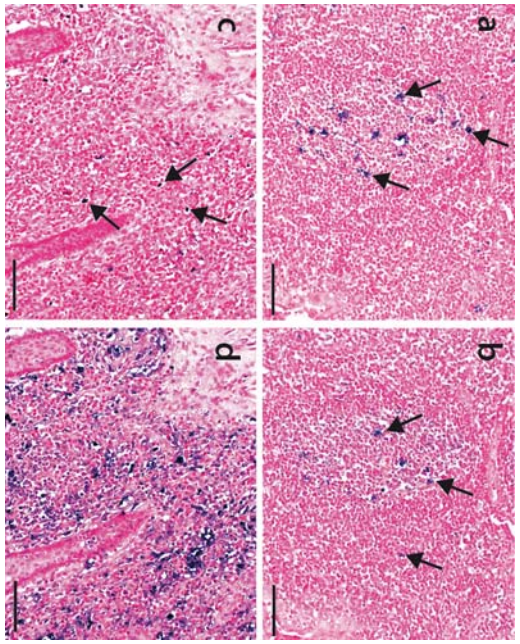
710 fixed and paraffin embedded tonsil tissue section. Red labelling achieved by hybridization of
711 PCV2a dsDNA specific oligonucleotide Ar (a). Oligonucleotide Br target hybridization specific
712 for PCV2b dsDNA appears green in the panel (b). Panel (c) is the result of pictures (a) and (b)
713 overlay. In all panels nuclei are counterstained with DAPI and thus appear blue. The bar
714 indicates 15 µm.

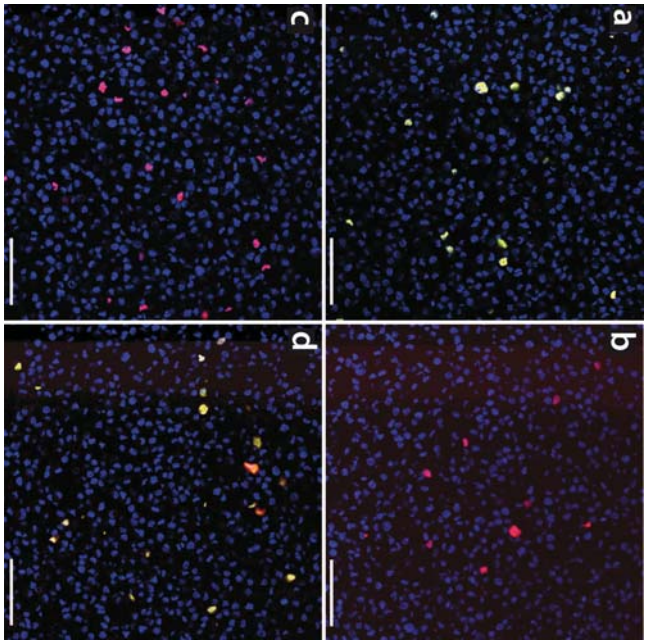


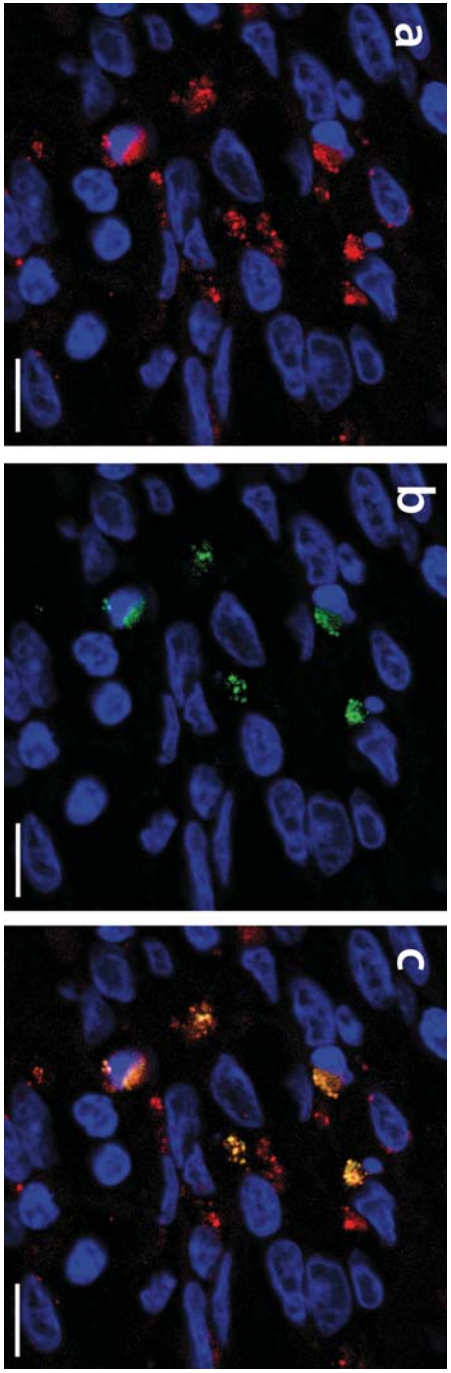


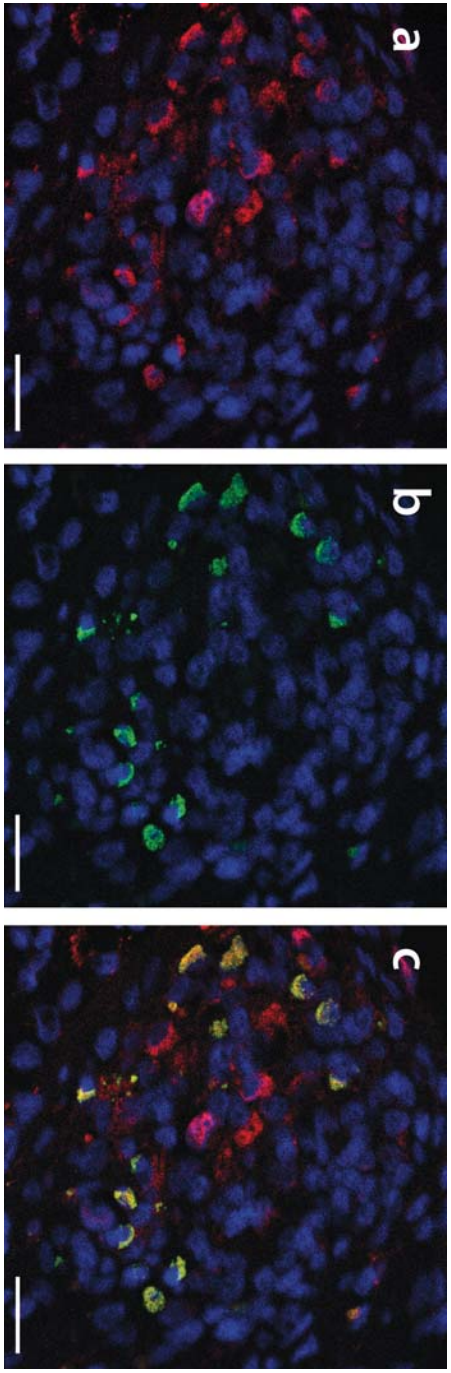


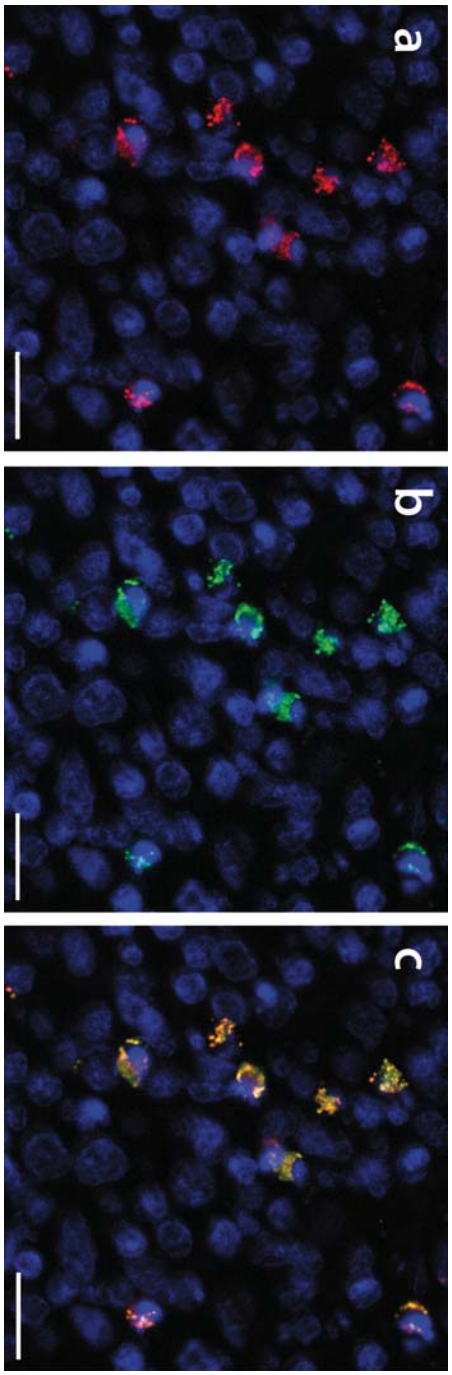







a
b
c


a
b
c


a
b
c